OXYGEN AND LUMINESCENCE, WITH A DESCRIPTION OF METHODS FOR REMOVING OXYGEN FROM CELLS AND FLUIDS.

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So many luminous animals require dissolved gaseous oxygen for luminescence that one might expect all to need it, but I have recently discovered certain forms which can luminesce without oxygen. Perhaps they utilize oxygen already bound up with the luminous material or perhaps the old question as to the storing of oxygen in cells must be reopened again. One should like to say, "Without oxygen neither life nor light." Such an epigram can only be true when, by oxygen, we refer to the element both free and combined. If we speak of anaërobic existence, we must also recognize anaërobic luminescence.

I arrived at this discovery through the observation that, although the luminescence of *Cypridina* is inhibited by strong light in presence of oxygen but not in its absence, the luminescence of *Mnemiopsis* and other Ctenophores is inhibited by light whether oxygen be present or not. This led me to reinvestigate methods of removing oxygen from fluids and the discovery that Ctenophore extracts can still luminesce under anaërobic conditions where other luminous forms (including *Cypridina*) show no trace of luminescence. It seems very probable, then, that in Ctenophores oxygen is firmly bound and that this is the reason we observe inhibition of their luminescence by light in absence (apparent) of oxygen. If oxygen is not bound in Ctenophores, their luminescence must result from a totally different type of reaction from that in other forms, for I believe the means employed for oxygen removal are without criticism.

[1] Appointed to the A. A. A. S. table at Naples, Oct. 1925–Jan. 1926.

There are four convenient methods of removing the last trace of oxygen from biological fluids. I say methods for *biological* fluids because none of these methods involves very great changes in H-ion concentration that might bring about changes in the fluid, although all of them are not suited for living cells. All these methods prevent the luminescence of bacteria (Harvey and Morrison, 1923) and *Cypridina*, which certainly means less oxygen than 10^{-5} atmospheres, and most of them involve nascent hydrogen.

1. By addition of cells actively using oxygen such as muscle, yeast or bacteria. If the fluid is a cell extract requiring oxygen, it will use up its own oxygen on standing in a narrow tube. The reducing power of cells varies but practically all will reduce methylene blue and most will reduce indigo-carmine. For the significance of this in terms of oxidation-reduction potential the reader is referred to the papers of Clark (1923–25).

2. Nascent hydrogen can be generated in the fluid by adding aluminium amalgam or magnesium plus some neutral ammonium salt. In the first case $Al(OH)_3$ is formed and in the second an ammonium-magnesium salt. In either case the P_H does not change much, and the oxygen is quickly washed out.

3. Small amounts of sodium hydrosulphite (or hyposulphite, $Na_2S_2O_4$, not thiosulphite) may be added to the fluid. It is best to dissolve the hydrosulphite in a powerful buffer and to buffer the fluid under investigation as the hydrosulphite is acid. To determine when the right amount of hydrosulphite has been added methylene blue can be used as indicator. It is decolorized when reduced by the hydrosulphite, which also absorbs all oxygen in the solution. Shaking with air or addition of potassium ferricyanide [$K_3Fe(CN)_6$] will oxidize the hydrosulphite with return of the blue color to methylene blue. The advantage of hydrosulphite is its practically instantaneous production of anaërobic conditions. The disadvantage is a possible injurious effect on living cells, which I believe should be investigated rather carefully. My own observations show that the ciliated cells of the gills of *Mytilus*, lightly stained in methylene blue, stop beating and the blue color disappears (reduction) in hydrosulphite sea water very quickly. When fresh aërated sea water

is added the blue color returns and then the cilia begin beating again.

The luminescence of a mixture of *Cypridina* luciferin and luciferase is immediately extinguished by hydrosulphite and returns again on shaking with air at a time when methylene blue is just beginning to show a slight blue color. If some dry powdered *Cypridinas* are added to hydrosulphite sea water, it will be observed that fragments of undissolved luminous material are luminescent but the light disappears very quickly. There is apparently some air entangled, possibly mechanically, with the dry photogenic substances and when this is used up luminescence ceases. I had previously made this observation in another manner (Harvey, 1920).

In using methylene blue as an indicator, it must be borne in mind that mere removal of oxygen will not reduce methylene blue but that once reduced, a blue color will return when traces of oxygen are present. However, the reoxidation of methylene blue is rather slow except in alkaline solutions. The color change of methylene blue is also affected by light (Clark, 1925). Absence of luminescence in *Cypridina* luciferin-luciferase or in luminous bacteria can also be used as an indicator of lack of oxygen (the former is more delicate; bacteria begin to luminesce at an oxygen pressure = .0053 mm. Hg) and mere removal of oxygen is sufficient for cessation of luminescence in both cases.

4. Addition of platinized asbestos to the fluid and passage of hydrogen through it. This affords a quite rapid method of removing oxygen from fluids and the hydrogen need not be absolutely free of oxygen. It is far more rapid than bubbling pure hydrogen through the fluid without platinized asbestos. By pure hydrogen I mean hydrogen that has been passed over platinized asbestos heated to dull redness in a quartz tube. Such hydrogen contains oxygen in equilibrium at 10^{-26} atmospheres. It must not come in contact with rubber or oxygen will be absorbed from the rubber in sufficient amount to cause luminescence of *Cypridina* luciferin-luciferase solution. My studies on luminescent solutions have impressed me with the difficulty of removing small amounts of oxygen from fluids and the slowness with which a fluid comes into equilibrium with a

gas. I believe the addition of platinized asbestos to the fluid through which hydrogen is passed will prove of great value to biologists in the study of cells under anaërobic conditions.

Instead of Pt asbestos, colloidal Pt or colloidal Pd may be used. The latter is conveniently prepared by adding to 5 cc. 0.2 per cent. $PdCl_2$ just enough dilute NaOH to make the $PdCl_2$ darken without giving a precipitate of basic $PdCl_2$, and then one drop of .042 per cent. hydrazine hydrate. The finely divided Pd adsorbs a good deal of hydrogen and will keep a solution free of oxygen for some time, as can be observed by using as indicator the luminescence of a mixture of luciferin and luciferase.

In order to show that the luminescence of Ctenophores, like *Beroë*, is independent of dissolved oxygen, it must be recalled that an extract made by squeezing *Beroë* through muslin and filtering through filter paper is non-luminescent itself, but gives a brilliant light on addition of fresh water, *dilute* salt solution, or various cytolytic substances like saponin, sodium glycocholate, ether, etc. Accordingly I tried the experiment of mixing *Beroë* extract made free of oxygen by $Na_2S_2O_4$ with $m/10$ Na_2HPO_4 solution, also made free of oxygen by $Na_2S_2O_4$. The Na_2HPO_4 was used instead of fresh water in order that the reaction would be alkaline enough to allow rapid coloration of leuco-methylene blue, added as an indicator, should any oxygen leak into the apparatus. This is shown in Fig. 1. In *A* is placed *Beroë* extract + $Na_2S_2O_4$

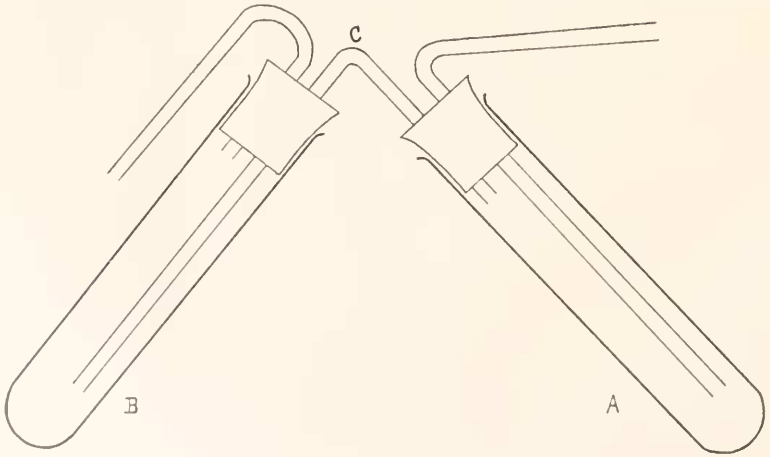

FIG. 1.

and in *B* $m/10$ Na_2HPO_4 solution ($P_H = 9$) + $Na_2S_2O_4$. The tubes allow a flow of pure hydrogen through a lead tube to wash out the air, and the only rubber parts are the stoppers of tubes *A* and *B*. In such an apparatus the contents of *A* and *B* can be mixed in hydrogen without the slightest blueing of leuco-methylene blue and oxygen-free *Cypridina* luciferin can be mixed with oxygen-free luciferase (by methods 3 or 4) without any luminescence appearing. However, when the oxygen-free *Beroë* extract (by methods 3 or 4) is mixed with the oxygen-free $m/10$ Na_2HPO_4 solution, just as bright a luminescence occurs, as in the control tests when mixed in presence of oxygen.

The experiment has been varied by using Pt asbestos in the *Beroë* extract and in the water and passing pure hydrogen through the system for an hour (although methylene blue can be decolorized in two or three minutes by the same flow of hydrogen), but a bright luminescence always results on mixing the *Beroë* extract with the water in the hydrogen atmosphere. When air is admitted and the fluid shaken, no further luminescence is to be observed.

The experiment has been repeated so often that I feel certain that Beroë extract will luminesce in absence of dissolved oxygen. *Eucharis multicornis*, another Ctenophore, behaves as *Beroë*, as does also the medusa, *Pelagia noctiluca*. *Pennatula phosphorea* extract, on the other hand, requires oxygen, as I had previously observed for the pennatulid, *Cavernularia haberi* Harvey (1917). A *Pennatula* extract in sea water mixed with fresh water in absence of oxygen [1] gives no light but when shaken with air the mixture luminesces faintly. It is somewhat surprising that the pennatulids should differ in this respect from the Ctenophores and *Pelagia* as the general aspects of the luminescence in these forms is similar.

None of these animals give the luciferin-luciferase reaction. The luminescence seems to be connected with the dissolution of granules. In the luminous slime of *Pelagia*, which sticks to the finger when rubbed over the exumbrella, can be observed with the microscope, cells densely crowded with granules somewhat less than 1 μ in diameter. These granules and some cell frag-

[1] Mixing in air of course results in luminescence.

ments will pass filter paper and centrifuging such a filtrate will throw down the cell fragments and many granules, leaving suspended only granules. When added to fresh water this centrifuged material gives a bright luminescence (because many granules are thrown down) while the suspension above gives a fainter luminescence. I could observe no cells in the suspension but when mixed with fresh water in complete absence of dissolved oxygen (method No. 4, hydrogen passed 20 mm.), as bright a luminescence occurred as when oxygen was present. I therefore believe that the oxygen is bound in these photogenic granules and that luminescence occurs upon their dissolution. That just enough or more than enough oxygen is so bound would be indicated by the fact that opening the tube containing the mixed fluids to the air results in no further luminescence.

This same experiment has been repeated with filtered and centrifuged *Beroë* extract, with the same result, luminescence of granular material in absence of oxygen.

The Radiolaria also require no free oxygen for luminescence. *Thalassicolla nucleata* and also colonies of *Colozoun inerme* mixed with platinized asbestos in sea water through which pure hydrogen is passed for 45 minutes will still luminesce whenever shaken. A colony of *Colozoun inerme* was broken up into separate cells (but these left uninjured) mixed with platinized asbestos and pure hydrogen passed through it for 20 minutes. It was then mixed in a pure hydrogen atmosphere with water and platinized asbestos through which pure hydrogen was passed for 20 minutes. A bright "starry" luminescence resulted, similar to that of *Beroë* extract when mixed with water.

After these rather startling (to me) results I thought it worth while to reinvestigate the necessity of oxygen for luminescence in other animals, noting carefully if the lack of oxygen caused luminescence to disappear when the whole animal was tested and when its secretion was tested. For we are especially interested in knowing whether the luminescence of dissolved or finely divided photogenic material is stopped by lack of oxygen rather than whether intact cells cease to luminesce in absence of oxygen. It is of course perfectly well known that luminous bacteria cease to luminesce in absence of oxygen but one could not make the

same statement of the *extract* of luminous bacteria for luminous material cannot be obtained apart from the living bacterial cell. The same is probably true of luminous fungi. They (*Panus stipticus*) require oxygen for luminescence. If ground in a mortar, *Panus stipticus* luminescence ceases almost immediately, so that an extract cannot be obtained.

The work of many investigators has shown that luminous *extracts* of *Pholas* (lamellibranch mollusc), *Cypridina* (ostracod crustacean), *fireflies*, *Cavernularia* (pennatulid), *Photoblepharon*,[1] *Anomalops*,[1] and *Malacocephalus* (fish) require oxygen for luminescence. I have recently investigated the following forms using extracts of the animals whenever possible.

Microscolex phosphorea, an earthworm producing a slime on stimulation, brightly luminescent with a yellowish light. The worm can be dried over $CaCl_2$ and gives a bright light on moistening with water. Worms shaken in pure hydrogen give no luminescence but produce a slime which luminesces immediately when air is admitted.

Chætopterus variopedatus, a polychæte producing a slime with bluish light on stimulation. In sea water the luminescence of the slime lasts long enough for one to observe the disappearance of luminescence when pure hydrogen is bubbled through it and the reappearance on admitting air.

Acholoë astericola, a polynoid worm whose scales emit a bright yellowish luminescence on stimulation. The light comes from groups of gland cells under the cuticula ending in a papilla with a pore, but not enough secretion is extruded to form a luminous solution. The whole animal when slowly heated in sea water or shaken gives a bright luminescence (over the scales) but if slowly heated or shaken in sea water with platinized asbestos through which hydrogen is passed, there is no luminescence.

Thelepus cincinnatus, a polychæte allied to *Polycirrus*, produces a luminescent slime on handling. When the whole worms are placed in a tube through which pure hydrogen is bubbled the luminescence disappears, but returns again when air is admitted.

Amphiura squamata, a brittle star whose arms produce a yellowish luminescence. The individual gland cells lie beneath

[1] The light of these forms is due to symbiotic luminous bacteria living in the organ.

the surface of certain plates and possess ducts that empty through pores in the cuticula, but not enough secretion is passed to the outside to give a luminous solution. The whole animals stimulated electrically in sea water plus platinized asbestos and air give a bright luminescence but no luminescence when stimulated in sea water plus platinized asbestos through which hydrogen has been passed for some time.

Balanoglossus minutus.—The whole of the skin produces a luminescent slime whose light does not last very long. If the whole animal is shaken in sea water through which pure H_2 is passing, there is no luminescence but on admitting air both worm and sea water show luminescence.

Copepods.—(Several species.) The cells are unicellular glands in definite positions in the body or in the legs, secreting to the exterior. When heated slowly in sea water with air the copepods give a bright luminescence but when heated slowly in sea water and platinized asbestos through which hydrogen was passed there was no luminescence. In one experiment the temperature was raised to boiling in the hydrogen atmosphere, then cooled and air admitted. The *solution* became faintly luminescent. A small amount of oxygen will cause luminescence and the hydrogen must be passed for some time to obtain anaërobic conditions.

Heteroteuthis dispar.—A squid in which a secretion from a gland (part of ink sac) is shot into the sea water as a bluish luminescent cloud of small granules often sticking together in clumps and filaments. On a microscope slide under a cover slip the secretion luminesces only at the edges in contact with air (but luminesces uniformly when the cover slip is lifted). When hydrosulphide is added to the secretion in a test tube the luminescence disappears except at surface but returns on shaking with air. Oxygen is necessary for luminescence.

Quatrefages observed luminescence of *Noctiluca* under what he regarded as anaërobic conditions whereas E. B. Harvey (1917) found the luminescence of *Noctiluca* to become very faint in a current of impure hydrogen (made in a Kipp generator but last traces of oxygen not removed) but to regain its brightness when air was admitted. It would seem that *Noctiluca* must require oxygen for luminescence. In view of my experiments with

Radiolaria and Cœlenterates it would certainly be worth while to test the luminescence of dinoflagellates, hydroids, siphonophoræ and medusæ in absence of oxygen. Many other higher forms await careful experimental investigation.

Summary.

Simple methods for completely removing oxygen from biological fluids are described and indicators for absence of oxygen discussed.

Most luminous animals require free gaseous dissolved oxygen for luminescence but a few can luminesce without such oxygen. These are the Ctenophores; the medusa, *Pelagia noctiluca;* and Radiolarians. Pennatulids require oxygen as do all annelida, ophiurians, cephalopoda, copepoda, and balanoglossids tested.

It is found that in *Beroë* and *Pelagia* the photogenic granules (without cells) luminesce in absence of oxygen, and it is suggested that the proper amount of oxygen for luminescence is bound up in the photogenic granule, and cannot be removed by the drastic methods of oxygen-removal herein described.

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